EXHIBIT C

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INTRODUCTION OF MACROMOLECULES INTO VIABLE MAMMALIAN CELLS
Renato Baserga, Carlo Croce, and Giovanni Rovera, *Editors*

INTRODUCTION OF MACROMOLECULES INTO VIABLE MAMMALIAN CELLS

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TRANSFER OF THE DIHYDROFOLATE REDUCTASE GENE INTO MAMMALIAN CELLS USING METAPHASE CHROMOSOMES OR PURIFIED DNA

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INTRODUCTION

Since the original report by McBride and Ozer '73, gene transfer systems have been developed for a number of somatic mammalian cell lines (McBride and Ozer, '73; Willecke, Lange, Krüger and Reber, '76; Wullems, van der Horst and Bootsma, '77; McBride, Burch and Ruddle, '78; Miller and Ruddle, '78; Wigler, Pellicer, Silverstein and Axel, '78; Wigler, Pellicer, Silverstein, Axel, Urlaub and Chasin, '79). Present transfer technologies, however, result in very low frequency of transfer (in the range 10^{-5} to 10^{-7}) and the variety of genes transferred has remained small. In addition, successful transfer, especially that mediated by purified DNA, has been confined to the use of the mouse L cell line as a recipient (Wigler, Pellicer, Silverstein and Axel, '78). Because of the large number of mutations characterized in the Chinese hamster ovary (CHO) cell line, these cells are particularly attractive for the application of gene transfer techniques. Using methods presently described in the literature (Spandidos and Siminovitch, '77(a); Miller and Ruddle, '78; Wigler, Pellicer, Silverstein, Axel, Urlaub and Chasin, '79), we were unable to achieve successful transfer in CHO. In order to improve transfer technology, we undertook a systematic study of the various steps involved in the transfer of the dihydrofolate reductase gene into mouse L cells. Here we report the results of these studies which have yielded a highly reproducible and efficient method for gene transfer into mouse and hamster cell lines.

MATERIALS AND METHODS

Cell Culture

Murine Ltk$^-$ cells (clone D) furnished by Dr. Silverstein were maintained in α-special medium (McBurney and Whitmore, '74) containing 10% fetal bovine serum (FBS). Chromosomes and DNA used in the gene transfer experiments were isolated from Mtx^{RIII}, a Chinese hamster ovary (CHO) cell line isolated in two steps for resistance to methotrexate (Flintoff, Davidson and Siminovitch, '76). The dihydrofolate reductase activity of Mtx^{RIII} has been well characterized biochemically. The first step selection resulted in cells which contained a dihydrofolate reductase activity more resistant than the wild-type enzyme to inhibition by methotrexate, and the second step resulted in elevated levels of the resistant enzyme. Because Mtx^{RIII} requires proline for growth it was grown in α-MEM medium (Stanners, Elicieri and Green, '71) which contains this amino acid. V79/V6 is a hamster lung cell line maintained in α-special medium.

Preparation of Chromosomes

Chromosomes were prepared by the procedure of Willecke and Ruddle (Willecke and Ruddle, '75) with some modifications. Mtx^{RIII} cells were grown in suspension to a density of 4 x 10^5 cells per ml and distributed in 50 ml aliquots into 20 flasks (150 cm^2). After 12 hrs at 37°C, in a CO_2 incubator, 3.5 μg of Colcemid (Sigma Chemical Co.) was added to each flask. After another 12 hrs of incubation at 37°C, the mitotic cells were detached by gently shaking 5 times. Approximately 90% of the detached cells were in mitosis. The cells were centrifuged at 200 g for 20 min, and resuspended in 50 ml cold hypotonic (75 mM) KCl. After 15 min at 4°C, the swollen cells were centrifuged at 200 g for 10 min. The pellets were resuspended in 40 ml of 15 mM HEPES buffer, pH 7.0, containing 3 mM $CaCl_2$ and 0.5% Tween 80 and transferred to a glass Dounce homogenizer. The cells were disrupted by 6 to 10 strokes of the homogenizer, and the suspension was centrifuged at 100 g for 8 min in plastic tubes to remove unbroken cells, nuclei and other debris. At this stage a sample of the supernatant was placed in a haemocytometer and viewed under phase contrast optics. The number of chromosomes was counted and expressed as cell equivalents per ml.

As outlined here, the isolation procedure yielded from 40 to 100 x 10^6 cell equivalents of chromosomes from 20 flasks of Mtx^{RIII} cells. The amount of DNA in the samples was occasionally checked by the Dische diphenylamine reaction, and this value correlated well with the amount of DNA expected on the basis of chromosome cell equivalents.

The supernatant solution of the low speed centrifugation was transferred to 4 siliconized 15 ml glass tubes and centrifuged at 1300 g for 20 min. The pellet in each tube was resuspended by agitation in 10 ml of 15 mM HEPES buffer, pH 7.0 containing 3 mM $CaCl_2$ and again centrifuged at 1300 g for 20 min. The washed pellets were resuspended at room temperature in 25 mM HEPES buffer, pH 7.1 containing 140 mM NaCl and 0.75 mM $Na_2HPO_4 \cdot 12\ H_2O$ at a concentration of 4 x 10^6 chromosome cell equivalents per ml. The buffered phosphate solution was prepared and the pH adjusted with 1N NaOH immediately before use.

Chromosome Transfer Method

The recipient Ltk$^-$ cells, logarithmically growing in stock flasks, were trypsinized and plated at 2 x 10^6 cells per 75 cm^2 flask containing α-special medium with 10% FBS. After 24 hrs, the medium was aspirated from the recipient flasks and 10 ml of fresh medium at 37°C added. To the chromosome preparation in HEPES-NaCl-phosphate buffer, $CaCl_2$ (2.5 M) was slowly added with mixing to a final concentration of 125 mM. One or two ml of this preparation was added immediately with a plastic pipette to the medium on the recipient cells. After an adsorption period in a 37°C CO_2 incubator, the medium was aspirated and 40 ml fresh medium added. When DMSO was employed, it was added to a final concentration of 10% directly into the chromosome-containing medium at the end of the adsorption period. After 30 min at 37°C in a CO_2 incubator, the medium containing DMSO was aspirated and replaced with 40 ml fresh medium.

Isolation of DNA

Six litres of Mtx^{RIII} cells were grown in suspension to a density of 5 to 7 x 10^5 cells per ml and centrifuged. The nuclei from these cells were isolated by swelling in hypotonic buffer followed by homogenization in a Dounce homogen-

izer. The crude nuclei were washed once with hypotonic buffer containing 0.5% Tween 80. The DNA from the nuclei was isolated essentially by the method described by Pellicer et. al., (Pellicer, Wigler, Axel and Silverstein, '78) and dissolved in 1 mM TRIS-Cl, pH 7.9 containing 0.1 mM EDTA. The concentration of the DNA was determined by the diphenylamine reaction.

DNA Transfer Method

The preparation of the calcium phosphate-DNA complex has been described (Wigler, Pellicer, Silverstein, Axel, Urlaub and Chasin, '79). In order to obtain reproducible results, we have introduced several modifications which are described in detail here. In a typical experiment, 160 µg of purified $Mtx^{R}III$ DNA was gently dispersed into a total volume of 3.6 ml of 1.0 mM TRIS-Cl, pH 7.9 containing 0.1 mM EDTA and stirred very slowly using a 1 cm teflon coated magnetic bar in a 50 ml siliconized round bottom flask. $CaCl_2$ (2.5 M) was added to give a concentration of 250 mM. Four ml of sterile HEPES buffer (50 mM), pH 7.1 containing 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 12\ H_2O$, prepared immediately before use, was introduced drop by drop along the side of the flask. After the addition, one or two ml of the precipitated solution was added immediately with a plastic pipette to recipient Ltk^- cells as described for chromosome transfer. After the adsorption period at 37°C, the medium was aspirated and replaced with 40 ml fresh medium.

Expression and Selection

The flasks were incubated at 37°C for an additional period of 40 hrs after the adsorption. The cells were then trypsinized and counted in a Coulter Counter. Thymidine kinase positive cells were scored by plating 1×10^6 cells in 100 mm plates in α-special medium containing 10% dialysed FBS, 10 µg/ml of hypoxanthine, 0.2 µg/ml of aminopterin, 5 µg/ml of thymidine and 50 µg/ml of glycine (HAT medium). After incubation at 37°C for 10 to 14 days, the medium was removed and the colonies stained with methylene blue. With this selection method, it was not necessary to change the HAT medium every 3 or 4 days and this prevented the formation of satellite colonies.

Methotrexate resistant cells were selected by plating 5×10^5 cells in 100 mm plates in α-special medium containing 10% dialysed FBS and 2×10^{-7} M methotrexate. The methotrexate solution was prepared immediately before use by dissolving the drug in 0.01 N NaOH. The concentration was estimated by using a molar extinction coefficient of 23,000 at 257 nm.

Controls

The results of transfer experiments are expressed as the number of transformant colonies produced when 10^7 cells are plated for selection. A number of controls were included in each experiment. No surviving colonies were seen when 10^8 untreated Ltk^- cells were plated in medium containing 2×10^{-7} M methotrexate. Similarly, control Ltk^- cells treated with chromosomes or DNA from Ltk^- or Mtx^S tk^- CHO, cells failed to result in any colony formation when over 10^8 cells were exposed to selective conditions.

COMMENTS ON METHODS

Preparation of Chromosomes

The method outlined here permits the isolation of large amounts of chromosomes in a reasonable time period and with a minimum of manipulation. Synchrony of the donor cells with either thymidine block or isoleucine starvation failed to significantly improve the yield of chromosomes. Hexylene glycol has been used previously for the isolation of chromosomes (Wray and Stubblefield, '70; Spandidos and Siminovitch, '77b). While the morphological integrity of chromosomes isolated in hexylene glycol is excellent, we have been unable to detect any gene transfer using such chromosomes. In addition, hexylene glycol treated chromosomes tend to be extremely sticky and therefore clumping and loss on glassware is a major problem. In our experiments, we have found that a neutral buffer containing low concentrations of HEPES and $CaCl_2$ to be suitable for chromosome preparation. Even with this isolation buffer, however, it is necessary to siliconize all glassware and to use only plastic pipettes while handling the chromosome preparations.

Because our chromosome preparations are not extensively purified, contamination by whole donor cells and nuclei does occur. In the experiments reported here, the recipient cells used do not require proline for growth, whereas the chromosomes were isolated from Mtx^{RIII} cells which are auxotrophic for this amino acid. Medium lacking proline was used during the expression and selection period to prevent the growth of any donor cells present in the chromosome preparation. Depending on the particular preparation, nuclear contamination on occasion has resulted in the formation of hybrid cell colonies at a low frequency ($<10^{-7}$). As a precaution, therefore, we screen transformant clones for hybrid karyotype and for the presence of additional genetic markers to rule out such hybrid formation.

It would facilitate gene transfer studies to be able to store chromosome preparations, but no data is available on the effect of storage on transfer frequency. In preliminary studies, several of our chromosome preparations, after the HEPES-$CaCl_2$ centrifugation step, were mixed with twice the volume of 40 percent glycerol and placed at -70°. At various time intervals, they were removed, diluted with an equal volume of HEPES-$CaCl_2$ buffer and centrifuged at 3,000 x g for 20 min. The pelleted chromosomes were resuspended in HEPES buffer containing NaCl and Na_2HPO_4 and used in transfer experiments after addition of $CaCl_2$. While the efficiency varied, the transfer frequency after storage for 4 weeks at -70° was 10 to 50 percent of that achieved with freshly prepared chromosomes.

Preparation of DNA

We have examined a number of batches of Mtx^{RIII} DNA for transfer of tk^+ and Mtx^R markers and the frequency of transfer of these genes varied at the most by two fold. The transfer efficiency did not diminish even though some of the DNA preparations were at least 40 million as estimated by agarose gel electrophoresis. Nevertheless, the frequency of transfer was only marginally decreased by using DNA sheared by passage three times through a 22 gauge, 1 1/2" needle.

Recipient Cells

Invariably satisfactory transfer was obtained when the recipient Ltk^- cells were plated from exponentially growing cells. Pretreatment of these cells with colcemid, colchicine and cytochalasin D as reported previously (Farber and Eberle, '76; Spandidos and Siminovitch, '77b; Miller and Ruddle, '78) not only caused considerable cell killing but also resulted in very poor transfer.

Calcium Phosphate Precipitation

Initially we encountered problems in achieving calcium phosphate precipitates which yielded reproducible transfer, especially that mediated by DNA. However, by preparing the HEPES-saline-phosphate solution immediately before use and by carrying out the mixing carefully during the precipitation step, this problem was overcome. Unexpectedly, we found that it was not necessary to allow the precipitate to form for 30 min, as described by others (Miller and Ruddle, '78; Wigler, Pellicer, Silverstein, Axel, Urlaub and Chasin, '79). In fact, immediately adding the precipitate to the recipient cell monolayer resulted in higher frequencies of gene transfer.

RESULTS

Of the various methods which have been used to facilitate gene transfer in mammalian cells, we chose the calcium phosphate co-precipitation technique first developed by Graham and van der Eb (Graham and van der Eb, '73) for the transfection of human KB cells with purified adenovirus DNA. The method has been used to successfully transfer several single copy mammalian genes via metaphase chromosomes or purified cellular DNA. A schematic diagram of the various steps involved in the transfer of the dihydrofolate reductase gene by the calcium phosphate co-precipitation technique is illustrated in Figure 1. In this system, gene transfer depends upon a number of variables including preparation and co-precipitation of chromosomes or DNA, adsorption period, gene dosage, treatment with adjuvants such as dimethylsulfoxide (DMSO) and expression time. In order to improve gene transfer frequency, we examined these parameters individually while keeping the others at near optimal levels based on preliminary study.

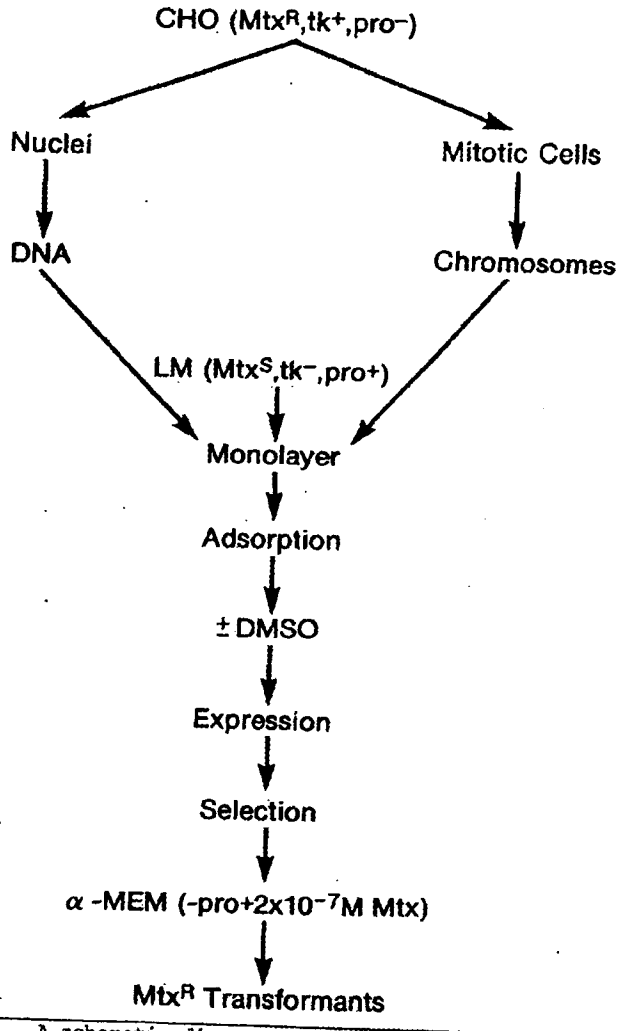

Figure 1. A schematic diagram of the various steps in the transfer of Mtx^R gene with metaphase chromosomes or with DNA.

Adsorption

While other workers have used relatively brief adsorption periods of 0.5 to 4 hours, we have found with all cell lines tested so far that longer adsorption periods yield significantly higher transformation frequencies. Results of an experiment where the adsorption period was varied from 4 to 20 hours for the transfer of the Mtx^{RIII} marker into Ltk$^-$ cells with chromosomes are shown in Figure 2. In the absence

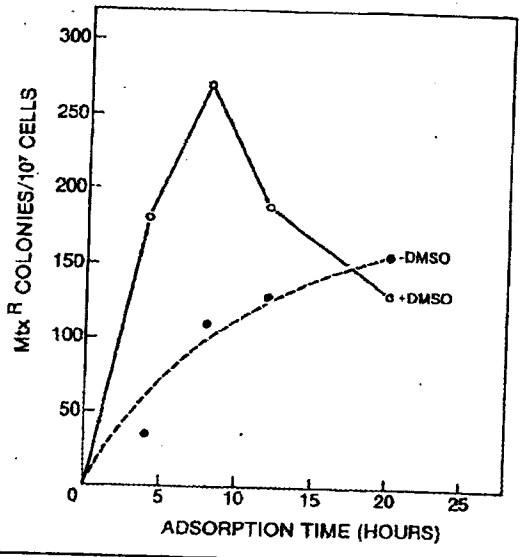

Figure 2. The effect of length of exposure of Ltk$^-$ cells to chromosomes from Mtx^{RIII} cells on the frequency of transfer of Mtx^R marker.

of DMSO treatment, the longer the adsorption time the greater is the yield of Mtx^R transformants. In this particular experiment, leaving the chromosomes in contact with the cell monolayer for 20 hours resulted in 160 methotrexate resistant colonies per 10^7 cells plated. Treatment of the recipient cells with DMSO, however, markedly enhanced the frequency of transfer, especially with short adsorption times. At 8 hours

adsorption, for example, DMSO treatment produced greater than 2 fold more Mtx^R transformants. In a number of experiments similar to that shown in Figure 2, the shape of the curves in both the presence and absence of DMSO treatment has varied considerably. However, using an adsorption period of 8 to 12 hours followed by DMSO treatment consistently has yielded the highest frequency of gene transfer, and we have chosen these conditions for routine experiments.

A representative experiment where the transfer of the Mtx^{RIII} marker was achieved with purified DNA as the transforming agent is shown in Figure 3. The frequency of transfer

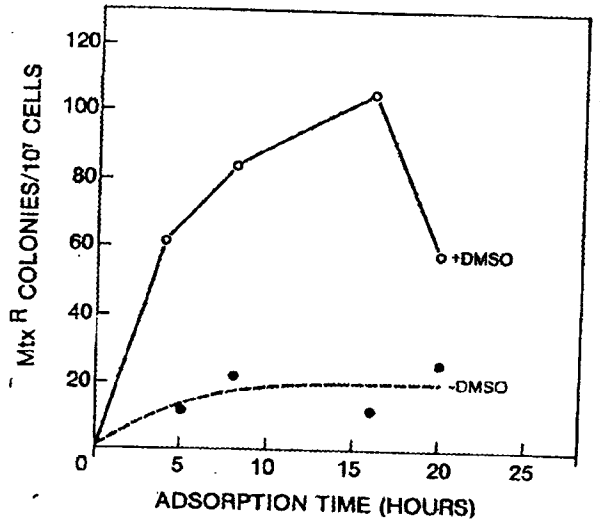

Figure 3. The effect of length of exposure of Ltk^- cells to DNA from Mtx^{RIII} cells on the frequency of transfer of Mtx^R marker.

was lower than that obtained with chromosomes as the gene transfer vector. With DNA, the effect of DMSO treatment on the number of Mtx^R transformants was striking; at all adsorption times a higher frequency of dihydrofolate reductase gene transfer was achieved after treatment with DMSO. For example,

at 8 hours adsorption a yield of only 20 transformants per 10^7 cells was increased 4 fold after treatment with DMSO. These studies suggest that an adsorption period of at least 8 hours followed by treatment with 10% DMSO is optimal for DNA mediated transfer of the dihydrofolate reductase gene.

Gene Dosage

The mechanisms underlying the efficient uptake of DNA or chromosomes in mammalian cells are not well understood. Table 1 shows the effect of increasing amounts of chromosomes or DNA upon the frequency of Mtx^R transformants, using an adsorption time of 8 hours with recipient L cells. With DMSO

Table 1. Frequency of Mtx^R gene transfer as a function of chromosomes or DNA dosage

	Mtx^R colonies/10^7 cells	
	Without DMSO	With DMSO[c]
Chromosome dosage[a]:		
a) 1×10^6	90	350
b) 2×10^6	530	710
c) 4×10^6	850	1350
DNA concentration[b]:		
a) 20 µg	0	28
b) 40 µg	10	43
c) 80 µg	7	76

a) The figures indicate cell equivalents of chromosomes added per flask of recipient L cells.
b) The figures indicate tho amount of DNA as determined by Dische diphenylamine reaction.
c) After adsorption for 8 hrs, a pulse of 10 percent DMSO for 30 min was given. For details see Materials and Methods.

treatment, increasing either the chromosome or DNA concentration resulted in a linear increase in the number of transformants. At a dosage of 4×10^6 cell equivalents of Mtx^{RIII} chromosomes per flask (approximately equal to 40 µg of DNA), the number of Mtx^R colonies formed was 1350 per 10^7 recipient cells; a frequency greater than one colony per 10^4 cells. Omission of DMSO treatment decreased this frequency by about 35%. With DNA, on the other hand, the frequency of transfer was poor in the absence of DMSO treatment and was not strictly

dependent on DNA concentration. Inclusion of DMSO markedly enhanced the transfer frequency, and at a dosage of 80 µg per flask a frequency of 10^{-5} was obtained. It is apparent from these experiments that on the basis of equivalent DNA contents the use of chromosomes results in 10 to 20 fold more transformants than when DNA is used.

DMSO Treatment

We have not examined in detail the effect of various concentrations of DMSO or various lengths of DMSO treatment. However, preliminary studies indicate that the frequency of transfer of the dihydrofolate reductase gene increases with increasing concentrations of DMSO (data not shown). Increasing the period of DMSO treatment to 60 minutes similarly resulted in an increase in transfer frequency. Using Ltk^- cells as recipients, concentrations of DMSO greater than 10% or exposure periods longer than 30 minutes, however, results in significant cell killing, and therefore we have continued routinely to use 10% DMSO for 30 minutes.

Expression Time

After the uptake of foreign genetic material, the sequence of events which allow for the expression of a transformed phenotype is not known. Presumably, at least some genetic markers may require a period in non-selective medium for formation of the gene product.

In the original experiments of McBride and Ozer (1973) mouse fibroblasts, after exposure to chromosomes from Chinese hamster cells, were allowed to grow in non-selective medium for 3 days before being plated in selective medium. More recently, Miller and Ruddle (1978) used an expression period of only 20 hours, and a similarly short expression period has been used by Wigler et al., (1979) for the transfer of the thymidine kinase gene into Ltk^- cells using DNA. To determine the effect of expression time on transfer of the dihydrofolate reductase gene, five separate flasks of Ltk^- cells were treated with 40 µg of Mtx^{RIII} DNA for 8 hours. Subsequently they were treated with 10% DMSO for 30 minutes. The DMSO containing medium from each flask was aspirated and fresh medium was introduced.

After 0, 15, 43, 66, and 94 hours in the non-selective medium, the cells were trypsinized and plated in 2×10^{-7}M methotrexate. After 2 weeks incubation, the number of Mtx^R colonies per 10^7 cells were 18, 28, 23, 20 and 2 respectively. Thus the frequency of transformation is more or less the same up to an expression period of 66 hours. The increased number of transformant colonies seen at 15 hours is probably not significant; other similar experiments have not shown this increase. After 66 hours in non-selective medium the number of Mtx^R colonies recovered decreased drastically and seemed to correlate with the resumption of rapid cell division. This decrease may reflect the instability of the transferred genetic material within the dividing cells.

Stability of the Transformed Phenotype

The size of the Mtx^R transformant colonies from chromosome transfer experiments was always considerably larger than that seen with DNA as the transforming vector. This is illustrated in Figure 4 where the left plate is from a chromosome transfer experiment and the right plate is from a DNA transfer experiment. With equivalent amounts of DNA, chromosome mediated transfer clearly yields more and larger colonies on the average than DNA mediated transfer.

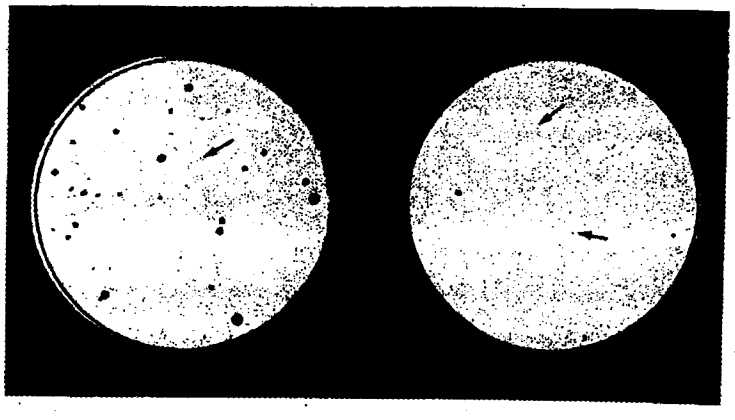

Figure 4. Left, Mtx^R colonies from a chromosome transfer

experiment. Right, Mtx^R colonies from a DNA transfer experiment. On each 100 mm plate, 5×10^5 Ltk^- cells that had been treated with 4×10^6 cell equivalents of Mtx^{RIII} chromosomes (left) or 40 µg of Mtx^{RIII} DNA (right) were plated in medium containing 2×10^{-7}M methotrexate. After 11 days incubation at 37°, the medium was removed and the colonies stained with methylene blue. The arrows indicate small colonies.

To test the stability of the methotrexate resistant transformant phenotype we picked at random 5 Mtx^R colonies from a chromosome transfer experiment and 12 Mtx^R colonies from a DNA mediated transfer experiment. None of the 12 Mtx^R colonies from the DNA transfer experiment, when grown for even a few generations in the absence of methotrexate, were able subsequently to form colonies in 2×10^{-7}M methotrexate, indicating their instability. The colonies picked from the chromosome transfer experiments behaved differently and were far more stable. Even after growth for 48 days in the absence of the drug, 2 clones out of the 5 isolated retained significant resistance to 2×10^{-7}M methotrexate. The remaining 3 transformant clones exhibited varying degrees of loss of the Mtx^R phenotype.

Transfer of the Thymidine Kinase Gene

A number of investigators have described transfer of the gene for thymidine kinase into mouse L cells using either metaphase chromosomes (Willecke, Lange, Krüger and Reber, '76; McBride, Burch and Ruddle, '78) or DNA (Wigler, Pellicer, Silverstein and Axel, '78) as transfer vectors. To determine whether the optimal conditions developed here for the transfer of the dihydrofolate reductase gene would also yield high frequency transfer for other genes, we attempted to transfer the thymidine kinase gene into recipient Ltk^- cells. Since Mtx^{RIII}, the donor cell line used in our studies, is both methotrexate resistant and possesses thymidine kinase, the transfer of both genes can be studied in parallel. Table 2 shows the result of treating Ltk^- cells with either Mtx^{RIII} chromosomes or DNA for a period of 8 hours, followed by a 30 minute exposure to 10% DMSO. After a subsequent 40 hour expression period, the cells were plated either in methotrexate or HAT containing medium. After 2 weeks incubation, the resulting Mtx^R or tk^+ transformant colonies were scored. Treatment of the Ltk^- cells with 4×10^6 cell equivalents of Mtx^{RIII} chromosomes resulted in approximately 1200 Mtx^R transformants per 10^7 cells. As Table 2 shows, an almost identical number of tk^+ transformants were obtained from the same experiment. In the control experiment chromosomes isolated from a tk^-, Mtx^S CHO cell line produced no transformant colonies under identical conditions.

Table 2. Comparative frequencies for the transfer of the genes for dihydrofolate reductase or thymidine kinase into mouse cells

Vector	Colonies*/10^7 cells	
	Mtx^R	tk^+
Mtx^{RIII} chromosomes	1230	1300
Mtx^S tk^- chromosomes	0	0
Mtx^{RIII} DNA	55	60
Mtx^{RIII} DNA minus phosphate	0	0
Mtx^{RIII} DNA minus calcium	0	0
Mtx^S tk^- DNA	0	0

* Figures indicate the number of transformant colonies obtained when 10^7 recipient cells are plated under selective conditions.

Also shown in Table 2, Mtx^{RIII} DNA used as the transfer vector resulted in equivalent numbers of tk^+ and Mtx^R transformants. In this case, however the number of transformants obtained was over 20 fold less than the number achieved with chromosomes as the transfer vector. If the formation of a calcium phosphate precipitation is prevented, either by omission of calcium or phosphate, then detectable transfer of either of the genes is abolished (Table 2).

Gene Transfer in Hamster Cell Lines

With the experience gained in determining the optimal conditions for efficient transfer of the dihydrofolate reductase gene into mouse L cells, we attempted transfer of this gene into the hamster cell lines V79 and CHO. Although we were able to achieve successful transfer with these cell lines, with both chromosomes and DNA, the frequencies were far less than those obtained when L cells were used as recipients. We have not varied the parameters here as extensively as with the mouse cells, but a typical experiment in which both the effect of adsorption time and DMSO treatment were examined is shown in Figure 5. Since the doubling time

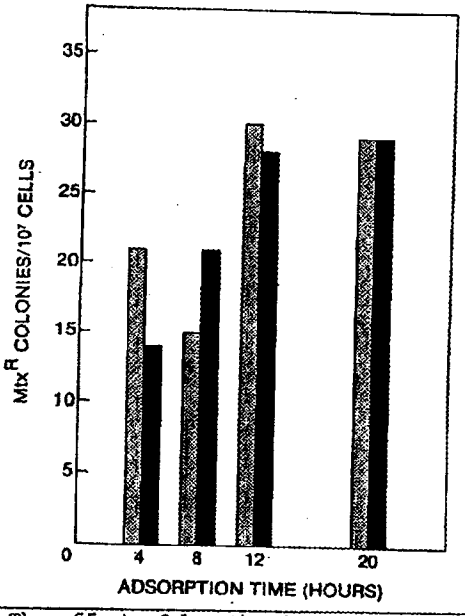

Figure 5. The effect of length of exposure of V79/V6 cells to chromosomes from Mtx^{RIII} cells on the frequency of transfer of the Mtx marker. ▒ minus DMSO, ■ plus DMSO.

of V79 is approximately 9 hours, recipient cells were plated at only 7 x 10^5 cells per flask 24 hours before addition of Mtx^{RIII} chromosomes. At 4, 8, 12 and 20 hours adsorption, the number of Mtx^R transformants per 10^7 recipient V79 cells varied from 15 to 30. While there appears to be no striking pattern, longer adsorption periods did yield more transformants. Figure also shows that under these transfer conditions DMSO had no significant effect on the frequency of dihydrofolate reductase gene transfer. The maximum values with V79 cells were therefore about 30 fold less than with L cells. Similarly low frequencies were obtained when the hamster thymidine kinase gene was transferred into V79 tk^- cells, both

with chromosomes and DNA (Table 3).

Table 3. Gene transfer frequencies for various recipient cell lines

Recipient	Vector	Mtx^R	tk^+
Ltk^-	chromosomes	1 x 10^{-4}	1 x 10^{-4}
	DNA	6 x 10^{-6}	6 x 10^{-6}
CHO	chromosomes	5 x 10^{-6}	1 x 10^{-7}
	DNA	2 x 10^{-7}	$<10^{-7}$
V79	chromosomes	3 x 10^{-6}	7 x 10^{-6}
	DNA	1 x 10^{-7}	2 x 10^{-7}

More recently, we have been able to transfer the dihydrofolate reductase and thymidine kinase genes with chromosomes or DNA into methotrexate sensitive CHO cells, using an adsorption period of 20 hours. The frequencies, however, are very low and work is in progress to improve the transfer method for this cell line.

Table 3 summarizes our results for gene transfer mediated by either chromosomes or DNA. Clearly, Ltk^- is a superior recipient cell line. The reasons for this are unknown. Table 3 also shows that irrespective of the gene transferred or the recipient cell line, the frequency of transfer is always significantly higher using chromosomes instead of DNA as the transforming vector.

CONCLUDING REMARKS

The results presented here indicate that three major parameters can affect transformation frequency. These are (a) adsorption time (b) gene dosage and (c) treatment with DMSO post adsorption. While DMSO has been used by Miller and Ruddle (1978) to enhance chromosome gene transfer its effect in L cells is far more impressive with DNA as the gene transfer vector. With L cells as recipients we have adopted the following conditions for efficient transfer by chromosomes or DNA as the agent: 8 hours adsorption period with a gene dosage of 40 µg of donor DNA or 4 x 10^6 cell equivalents of chromosomes followed by a 10 percent DMSO treatment for 30 minutes and an expression time of 40 hours.

As pointed out earlier we have consistently found a much

higher frequency for transfer of the Mtx^R marker when we have used chromosomes. Under similar conditions of adsorption and DMSO treatment, in 5 separate experiments the frequency of chromosome transfer of the Mtx^R marker was 1.5×10^{-4}, whereas that for DNA transfer was 6.4×10^{-6}. The lower frequencies with DNA may be due to unstable integration or abortive transformation.

Successful gene transfer with Chinese hamster cells as recipients has been difficult in our own and other laboratories. With the methods described in this paper, we have been able recently to transfer the genes for leucyl tRNA synthetase (Thompson, Harkins and Stanners, '73) and ribonucleotide reductase (Lewis and Wright, '78; Lewis and Wright, '79) into Chinese hamster cells by chromosomes albeit with very low frequencies. The availability of a large spectrum of well characterized mutants in the CHO cell line, and now a reliable gene transfer method, augurs well for future genetic studies with this cell line.

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